Antagonistic dark/light-induced SigB/SigD, group 2 sigma factors, expression through redox potential and their roles in cyanobacteria

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Abstract The expression of group 2 sigma factors is characterized in a cyanobacterium *Synechocystis* sp. PCC 6803 grown in culture, changing light conditions (white, red and blue light, and darkness), or the presence of drugs (rifampicin, chloramphenicol, DCMU, and DBMIB), and the roles of these sigma factors are elucidated. The expression of dark/light-induced SigB/SigD was accelerated under opposite redox (oxidation/reduction) states in an electron transport chain of photosynthesis. Expression of the dark-induced *lrtA* and light-induced *psbA2/3* transcript was significantly reduced in the *sigB* and *sigD* knockout strains, respectively. Abundant amounts of *sigB* transcript and protein were observed in the *sigC* knockout strain, implying that SigC represses SigB expression under light. These findings clearly showed that SigB/SigD with another group 2 sigma, SigC, contribute to transcription for a subset of dark/light-responsive genes in the cyanobacterium. A possible model for SigB/SigD is presented and the potential ability for promoter recognition is also discussed.

Key words: Sigma factor; RNA polymerase; Light/dark response; Redox

1. Introduction

The RNA polymerase holoenzyme of eubacteria consists of a core enzyme and a sigma factor [1]. The core enzyme is capable of undergoing transcriptional elongation and the sigma factor is required for the initiation of transcription from a specific promoter sequence. A number of sigma factors are usually encoded by a bacterial genome and sigma factors have been classified into three groups [2]. Group 1 comprises principal sigma factors that are responsible for transcription from a number of housekeeping promoters and are eventually essential for cell viability. Group 2 sigma factors are similar to the group 1 types in molecular structure, but are non-essential for cell viability. Group 3 sigma factors are an alternative type, structurally different from proteins of group 1 and group 2, and are sometimes involved in the transcription of regulons for survival under conditions of stress.

Cyanobacteria are prokaryotes that can perform oxygenic photosynthesis like plants and algae. Nine sigma factor homolog genes have been identified by genome analysis in the cyanobacterium *Synechocystis* sp. strain PCC 6803. The classification of PCC 6803 sigma factors, based on previous data [2–6] and phylogenetic analyses with the software Clustal W [7], have shown multiple group 2 and 3 sigma factors (Table 1). Although PCC 6803 is a non-nitrogen-fixing cyanobacterium, no RpoN-type sigma factor has been identified even in nitrogen-fixing cyanobacteria (e.g. *Anabaena* sp. strain PCC 7120) [16–18]. Clarifying the mechanism for light-responsive expression is one of the priorities in the study of photosynthetic organisms. A light-induced transcript of *psbA*, which encodes a key D1 protein in photosystem II (PS II), has been well characterized in cyanobacteria. The functional *cis*-elements and possible *trans*-acting factors contributing to the expression of *psbA* have also been identified [19–24]. However, few genes exhibiting dark-induced transcription have been identified [25–27]. Although the mechanisms of light- or dark-responsive gene expression have been discussed, our understanding of the light-responsive sigma factors and their roles in regulation is still limited. Recent study has revealed that PCC 6803 SigD is a high-light-induced sigma factor and specific *psbA2* promoter recognition was found in vitro [3]. Neither a dark-responsive sigma factor nor the biological relevance of such a factor has been reported to date. In this study, we identified dark/light-induced PCC 6803 group 2 sigma factors whose expression was modified by the redox potential of the electron transport chain in photosynthesis. We also present evidence of their contributions to dark/light-responsive gene expression in PCC 6803 cells.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The PCC 6803 cells, whose original wild-type strain was obtained from Kazusa DNA research institute, were grown at 30°C with shaking (120 rpm min^{-1}, NR-30, Taitec, Tokyo, Japan) in 100 ml of BG 11 medium [28] under 35 μmol m^{-2} s^{-1} of white light in a growth chamber (CU-255, Tomy Seiko Co. Ltd., Tokyo, Japan), supplemented with 15 μg ml^{-1} of kanamycin sulfate if required for the sigma knockout strains [3]. For red light (λ_{max} = 660 nm) and blue light (λ_{max} = 470 nm) experiments, PCC 6803 cells were incubated under illumination by panels (LED-R/B, Tokyo Rika Co. Ltd., Tokyo, Japan) in the growth chamber. The irradiation was quantified using a photodiode connected to a calibrated quantum sensor (LI-250 Quan-

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Abbreviations: DCMU, 3-(3′, 4′-dichlorophenyl)-1,1′-dimethylurea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone

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tum Sensor, LI-COR Biosciences, NE, USA). For drug treatments of the cells, rifampicin (Rif), chloramphenicol (Cm), 3-(3′, 4′-dichlorophenyl)-1,1′-dimethylurea (DCMU), and 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) were added to the medium at a final concentration of 200 μg ml^{-1}, 250 μg ml^{-1}, 5 μM and 5 μM, respectively.

2.2. Western blot analysis

Details were described in a previous report [3]. Each sample of total protein (35 μg) was subjected to an analysis with specific anti-sigma or anti-RpoB antibodies.

2.3. Primer extension analysis

Primer extension analysis was performed as described previously [29]. The oligonucleotides used in the primer extension for *sigB*, *lrtA*, and *psbA2* were as follows: sigB-R2 [3]; lrtA-R4, 5′-AAAACTAAGTTAGATTGGGC-3′; psbA2.3-R2, 5′-GCTTTCGCGCTGTTGGAGAG-3′. The oligonucleotides used in the primer extension for *psbA3* were the same as those for *psbA2*, psbA2.3-R2. The sequencing ladders (A, C, G, and T) were obtained with the same primers and relevant fragments, containing the promoter region of each gene cloned into pUC119B [30]. The plasmids and the regions are as follows: pYS0306 (−477 to +58), pLRTA (−497 to +40), and pSBA2 (−451 to +79) for *sigB*, *lrtA*, and *psbA2*, respectively. The transcriptional start point of *psbA3* was identified as described previously [31].

3. Results

3.1. Light- or dark-induced sigma factors

We examined the protein level in PCC 6803 of all sigma factors in light or darkness to identify light- or dark-inducible types by Western blot analyses (Fig. 1). When PCC 6803 cells were exposed to light after the adaptation to darkness, the SigD level significantly increased at 1 h (about a 2.5-fold increase compared with 0 h) and this persisted for 9 h (Fig. 1A). The amount of SigE gradually increased after 3 h, and reached a peak at 9 h (approximately 10 times that at 0 h). In contrast, the amount of SigB decreased under light, the level being approximately 25% (9 h) of that at 0 h. Interestingly, SigB significantly increased two-fold (9 h vs. 0 h) and SigE decreased (about 65% down at 9 h) under darkness (Fig. 1B). SigA, SigC, and RpoB levels were almost constant under both conditions. No group 3 sigma factor was detected under either set of conditions, as reported previously [3]. These results indicated that SigD and SigE are quick and slow responsive light-induced sigma factors, respectively, while SigB is a dark-induced sigma factor.

3.2. Sensitivity of SigB/SigD expression to drugs, redox, and blue/red light

We further examined the sigma levels in the PCC 6803 cells grown with drugs: Rif (an inhibitor of transcription), Cm (an inhibitor of translation), and two herbicides of DCMU [an inhibitor of electron transport between the PS II complex and the plastoquinone pool (PQ)] and DBMIB (an inhibitor of electron transport between the PQ and the cytochrome b_6f complex), respectively (Fig. 2A). Interestingly, SigB levels did not decline on addition of Rif even in the light (lanes 2 vs. 3), but were reduced by Cm (lanes 2 vs. 3 and 4), suggesting a downregulation of SigB expression which involves inhibition by de novo synthesized RNAs under light. This point is examined further in the next section. Although both SigD and SigE are identified as light-induced sigmas (Fig. 1A), we investigated the effects of the drugs on SigD expression this time. We have confirmed a prompt response of light/high-light-induced *psbA* gene expression [21,22], and SigD specifically recognized the *psbA* promoter in vitro [3]. Under light, the amount of SigB was greater in the cells treated with DCMU or DBMIB (DCMU < DBMIB) than those not treated with the drugs (Fig. 2A, lanes 2 vs. 5 and 6) and also significantly increased under darkness (general oxidative condition in the electron transport chain of photosynthesis) (Fig. 1). These phenomena suggested that the 'oxidative state of the components downstream' of the PQ pool induces the SigB synthesis (see Section 4 and Fig. 5). On the other hand, the amount of SigD was also increased in the presence of DCMU or DBMIB (DCMU > DBMIB; Fig. 2A, lanes 2 vs. 5 and 6) and an apparent increase of SigD was observed under light (reductive condition) (Fig. 1), suggesting that the 'reductive state of the components upstream' of the PQ pool induces the SigD synthesis. The efficacy to the increase in SigB by DBMIB (> DCMU) or SigD by DCMU (> DBMIB) seems to depend on the site of action of the drugs in the electron transport chain, with more oxidative conditions caused by DBMIB for SigB and more reductive conditions caused by DCMU for SigD (see Fig. 5). Addition of Cm

Table 1
Classification and relationship of functional sigma factor genes in *E. coli* and *Synechocystis* PCC 6803

Group	*E. coli*		*Synechocystis* PCC 6803		
	Gene	Function	Gene	Possible function	Reference
1	*rpoD*	Housekeeping	*sigA* (*slr0653*)	Housekeeping	[3,4,6]
2	*rpoS*	Stationary phase specific, multi-stress response	*sigB* (*sll0306*)	Dark and heat-shock response (oxidative stress)	[3,4,8–10], this study
			sigC (*sll0184*)	Post-exponential phase activated	[3,4,11]
			sigD (*sll2012*)	Light/high-light response (reductive stress)	[3,4,8,9], this study
			sigE (*sll1689*)	Nitrogen metabolism?	[3,4,12,13]
3	*rpoF*	Flagellar and chemotaxis	*sigF* (*slr1564*)	Motility	[3,14,15]
	rpoH	Heat-shock			
	fecI	Ferric citrate transport (ECF)	*sigG* (*slr1545*)	?	[3,15]
	rpoE	Extreme heat-shock (ECF)	*sigH* (*sll0856*)	?	[3,15]
			sigI (*sll0687*)	?	[3]
RpoN	*rpoN*	Nitrogen metabolism	No related gene	–	

ECF: extracytoplasmic function.

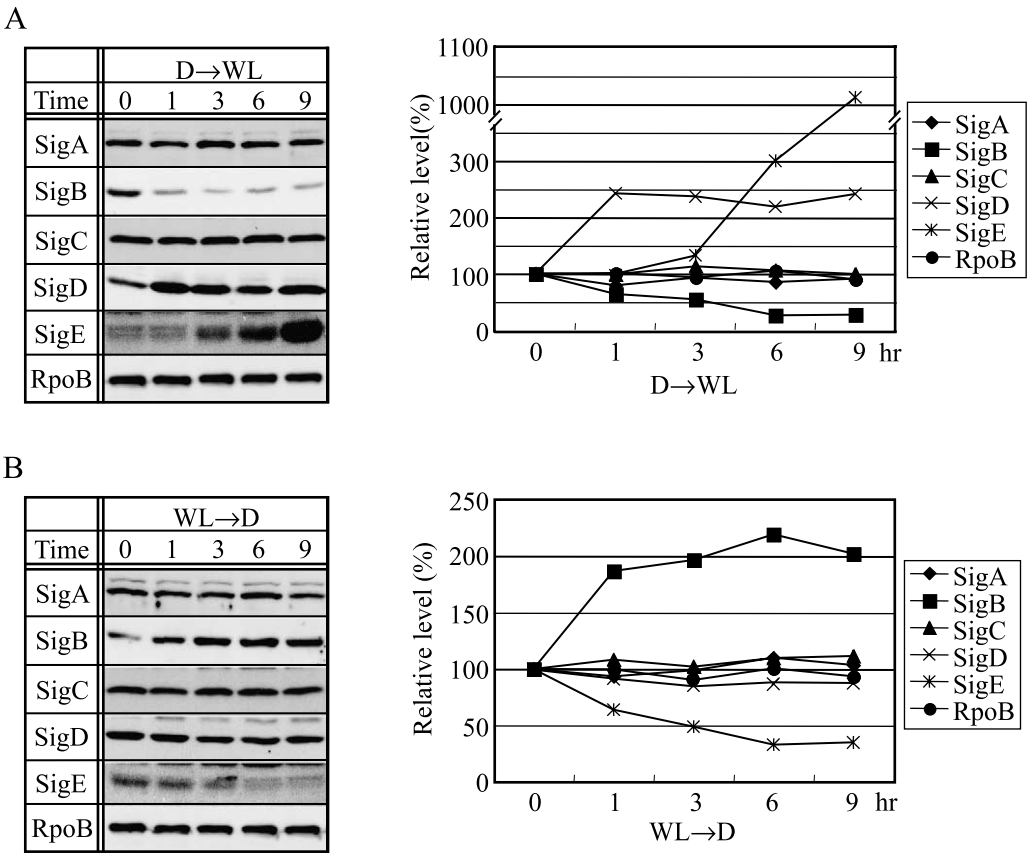


Fig. 1. Light/dark-induced sigma factors. A: PCC 6803 wild-type cells were grown under white light (WL, 35 μmol m^{-2} s^{-1}) until the mid-exponential phase [3] and incubated in complete darkness (D) for 12 h, then the lights were turned on again (D→WL). The cells were sequentially harvested at the time (h) shown in the figure. Aliquots of total protein from the cell lysate were subjected to Western blotting (left). The signal intensities were quantified as described previously [3]. The values from time 0 are normalized as 100 for each component (right). B: The PCC 6803 cells were grown under continuous WL until the mid-exponential phase and then incubated in complete darkness (WL→D). Other conditions are the same as in panel A.

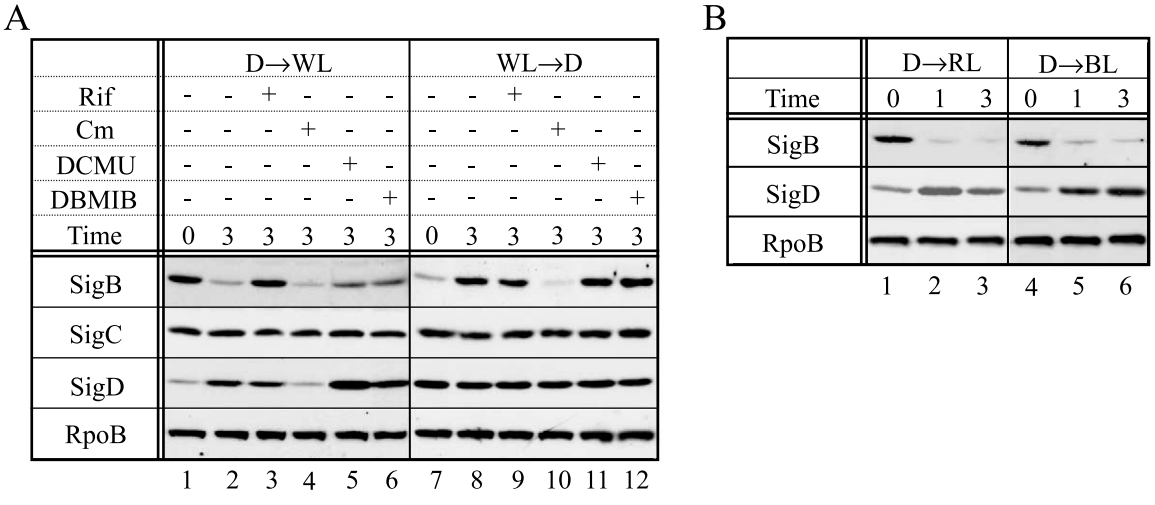


Fig. 2. Effects of drugs and light on SigB and SigD expression. A: The PCC 6803 cells were grown under the same conditions as shown in Fig. 1, with Rif, Cm, DCMU, or DBMIB added at 0 h, then the level of each protein was examined by Western blotting. The presence (+) or absence (−) of the respective drug is indicated. B: The PCC 6803 cells were grown under the same conditions as shown in Fig. 1A, then red light (RL, 20 μmol m^{-2} s^{-1}) or blue light (BL, 20 μmol m^{-2} s^{-1}) was irradiated for 0–3 h after the darkness (12 h). Each protein was also analyzed by Western blotting.

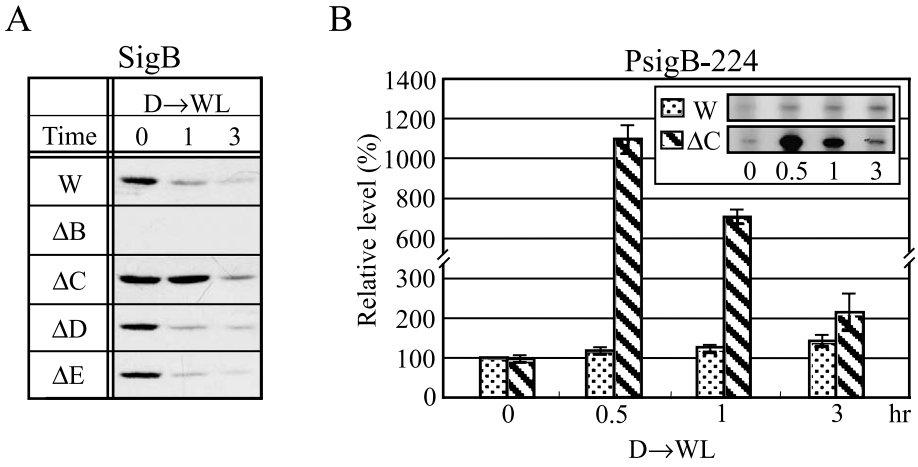


Fig. 3. Increased protein and transcript levels of *sigB* in *ΔsigC* cells. A: The PCC 6803 cells of wild-type (W), and *sigB* (ΔB), *sigC* (ΔC), *sigD* (ΔD), and *sigE* (ΔE) knockout strains were grown under the same conditions as shown in Fig. 1A, then the amount of SigB protein was examined by Western blotting. B: Under the same conditions as in panel A, total RNA (8 μg) was isolated, and primer extension with the sigB-R2 primer was conducted. A profile of the transcripts of PsigB-224 in the W or ΔC cells is shown as an inset. Signal intensities on X-ray films from three independent experiments were quantified the same as in Fig. 1 and the values were presented ($n = 3$, means ± S.D.) as relative levels (0 h of W as 100%).

prevented the dark/light-induced SigB/SigD expression, however, the level was not reduced when Rif was added, implying that the inductions were not substantially regulated at the level of transcription. SigB and SigD also exhibited reduced or enhanced expression under red/blue light irradiation (Fig. 2B), as under white light irradiation (Fig. 1). From these results, SigB/SigD expression might be controlled by multiple steps in a process which senses redox signals probably linked to a specific wavelength of light.

3.3. SigB expression repressed by SigC

To further characterize the downregulated SigB expression mentioned above (Fig. 2A), we measured the amount of SigB in each group 2 sigma knockout strain [3] (Fig. 3A). Surprisingly, a large amount of SigB still remained specifically in the *sigC* knockout strain (Δ*sigC*) during 3 h. Next, the *sigB* transcript was analyzed by primer extension in the Δ*sigC* cells (Fig. 3B). As in our previous study, *sigB* possessed four putative transcription start points (TSPs) which are mainly transcribed from PsigB-224 (> 95%) in the exponential phase [3]. The transcript levels were dramatically elevated, being approximately 11 times higher than in the wild-type at 0.5 h after the shift to light. These results clearly indicated that SigC directly or indirectly represses the expression of *sigB*.

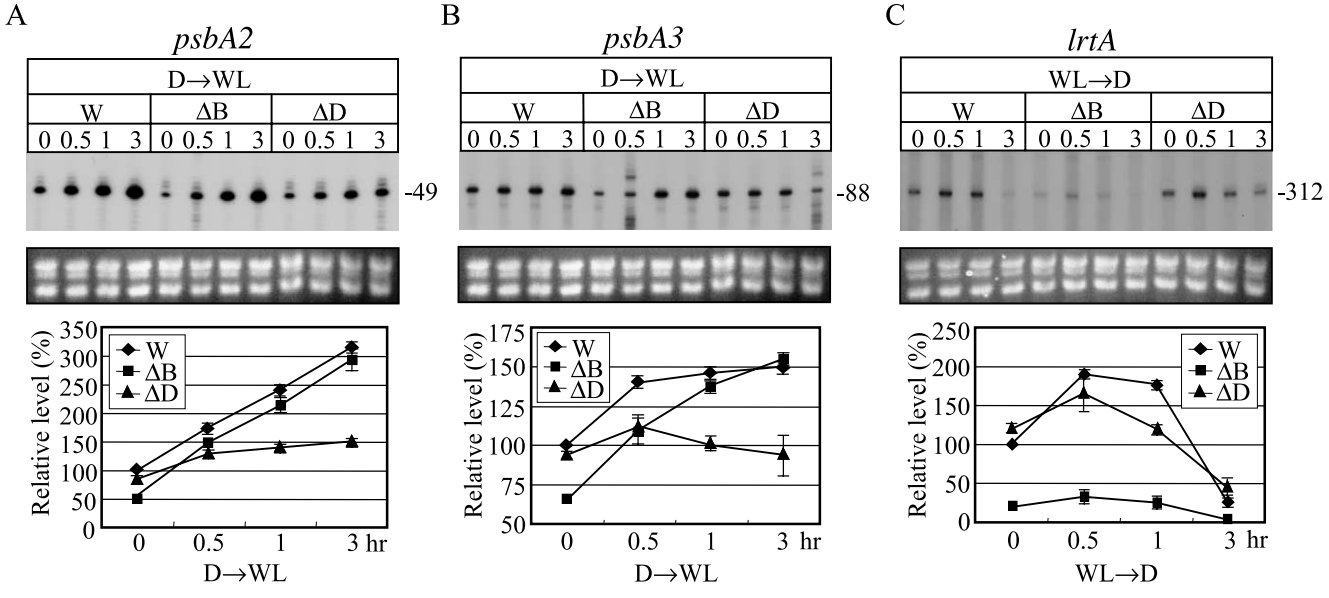
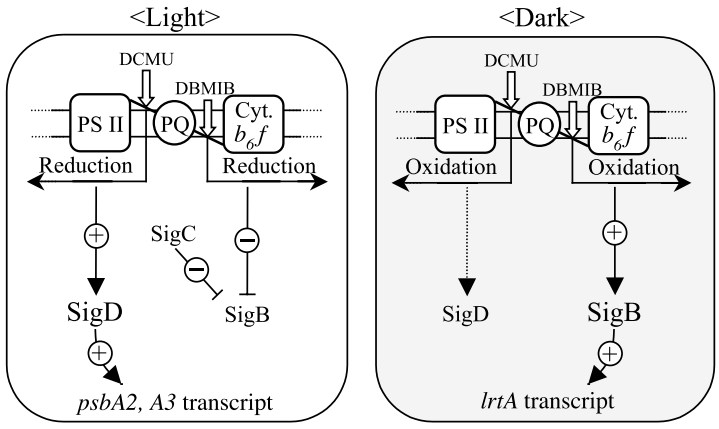


Fig. 4. Decreased light/dark-responsive *psbA*/*lrtA* transcription in the *ΔsigD*/*ΔsigB* cells. A,B: Under the same conditions as shown in Fig. 1A, transcripts of *psbA2* (A) and *psbA3* (B) were analyzed by primer extension with the primer psbA2,3-R2. The 5′-end position of each transcript (PpsbA2-49, PpsbA3-88: +1 as the initiation codon) is shown at the top. Total RNA (6 μg) used in the primer extension was resolved by gel electrophoresis (1.2% agarose/2.5% formaldehyde) and a part of the gel profile (23S and 16S rRNA) is presented in the middle as an RNA loading control. The signal intensities of the results of primer extension were quantified the same as in Fig. 3B, and are also presented at the bottom ($n = 3$, means ± S.D.). C: Transcripts (PlrtA-312) of *lrtA* in the WL → D condition were analyzed by primer extension with lrtA-R4. Others are the same as in panels A and B.

A



B

Condition	PS II (upstream)	PQ	Cyt. b_6f (downstream)	SigD	SigB
Light	Rd	Rd	Rd	Up	Down
Dark	Ox	Ox	Ox	N.C.	Up
+ DCMU	Rd	Ox	Ox	Up	Up
+ DBMIB	Rd	Rd	Ox	Up	Up

Fig. 5. A possible model for the contribution of SigD/SigB to light/dark-induced gene expression. A: Redox state (reduction in light, oxidation in darkness) is presented with the electron transport chain of photosynthesis. Antagonistic SigB/SigD expression through the redox state in darkness or light and inhibition by SigC of the SigB expression are presented in possible signal transduction pathways involving positive (+) and negative (−) effects. Details are described in the text. The sites of inhibition by DCMU and DBMIB of electron transport are represented by open arrows. B: The redox state of components of the electron transport chain of photosynthesis under several conditions is summarized. Correlative relationships between redox state and up/downregulated expression of SigD/B are presented with open squares. Rd, reductive state; Ox, oxidative state; N.C., almost no change; Up, induced expression; Down, reduced expression.

3.4. Contribution of SigB/SigD to light- or dark-induced transcription

We investigated whether SigB/SigD actually contributes to dark- or light-induced expression in PCC 6803 cells (Fig. 4). This time, we chose as representative genes, light-induced *psbA2* (*slr1311*) and *psbA3* (*sll1867*) for SigD, and dark-induced *lrtA* (*sll0947*), which has been characterized as a dark-responsive gene in *Synechococcus* sp. strain PCC 7002 [25], for SigB. In the case of *psbA2*, the amount of transcript remarkably decreased (about 50% at 3 h, Δ*D* vs. *W*) under light and the light-induced expression was almost destroyed in Δ*sigD*, whereas a clear pattern of induction was still observed in the Δ*sigB* cells (Fig. 4A). We obtained a similar result for the *psbA3* expression (Fig. 4B). These results clearly showed that light-induced SigD expression contributes to the light-induced *psbA2/3* transcription in PCC 6803 cells. On the other hand, we confirmed that the dark-responsive *lrtA* transcript (Fig. 4C, W), the 5′ end of which is at position −312 (+1 being the initiation codon), and PlrtA-312 possesses only the −10 hexamer, ATTAGGTCTTATTCAATACA-TAGTGCTAATCTGAAGATa: the putative −10 and TSP are indicated by an underline and small letter, respectively. The dark-induced transcript in Δ*sigD* behaved the same as that of the wild-type, however, the *lrtA* transcript had almost disappeared in Δ*sigB* (Fig. 4C, ΔB). This clearly showed that SigB is a dark-induced sigma factor, which contributes to the transcription of *lrtA* in PCC 6803 cells. Of note, the amount of *psbA2/3* transcripts was slightly lower in Δ*sigB* than in the wild-type under darkness (Figs. 4A,B, 0 h). This phenomenon does not necessarily contradict our finding that SigB is a dark-responsive sigma factor.

4. Discussion

This is the first study showing evidence of functional sigma factors for dark- and light-inducible gene expression in photosynthetic organisms. A possible model is presented in Fig. 5A. A summarized schema for the redox state of the components is also shown in Fig. 5B that indicates a correlation of reduction/PS II (upstream) vs. SigD induction or oxidation/cytochrome b_6f (downstream) vs. SigB induction. This suggested that the 'redox state of the components downstream and upstream of the PQ pool' influences the synthesis of SigB and SigD, respectively. Under light (reductive state), SigD increases and contributes to the light-induced *psbA2/3* gene expression, while SigC represses the SigB expression. In darkness (oxidative state), SigB increases and contributes to the dark-induced *lrtA* gene expression. SigB may be a sigma factor, expressed under oxidative conditions, because SigB protein levels also increase following heat-shock [3], which may result in an oxidative state owing to the denaturation of PS II [32]. Previous studies have suggested that the redox state of PS II seems to be involved in the regulation of light-induced *psbA* expression in cyanobacteria [9,33,34]. On the other hand, it has been reported that the redox state of components between the PQ pool and PS I regulates some light-dependent and light-independent gene expression [35,36]. In contrast, transcriptional regulation of photosynthesis-related genes based on the 'redox state of the PQ pool' has been indicated in green algae and higher plants [37,38]. In *Synechocystis* PCC 6803, the amount of *sigB* and *sigD* transcripts significantly increased in darkness and high light, respectively [8,10]. The *sigB* and *sigD* transcripts were induced by both DCMU and

DBMIB, and *sigD* transcripts substantially increased in the high-light and light conditions [9]. These phenomena also do not contradict the results of the present study on protein levels, and support well our possible model (Fig. 5A). Although the direct or indirect repression of *sigB* expression by SigC is of interest (Fig. 3), the mechanism involved is still unknown. How does SigC hinder these processes? It is known that the translation of *rpoS* (a group 2 sigma gene, Table 1) is inhibited by small RNAs of OxyS in *Escherichia coli* [39]. PCC 6803 may have same regulational system for SigB expression with small RNAs transcribed by SigC.

In this study, we also showed the specific contribution of SigB/SigD to dark/light-induced gene expression in vivo (Fig. 4). The possible PCC 6803 *lrtA* promoter has a canonical −10 hexamer but not the −35 elements, like the *sigB* promoters (PsigB-90, PsigB-64, and PsigB-11) which are recognized by SigB [3]. Taking all these things into consideration, SigB may recognize only the −10 type promoter. On the other hand, the principal sigma factor, SigA (group 1), also exists with SigB/SigD in the cells in darkness/light (Fig. 1, [3]). In this situation, how do SigB/SigD (group 2) specifically contribute to the transcription? Overlapping and distinct promoter recognition in vivo and/or in vitro among several sigma factors on *E. coli* consensus-type promoters were reported in not only cyanobacteria [3,4,22,40] but also higher plants [41]. *psbA* promoters, of *E. coli* consensus-type, are well recognized in vitro by principal sigma factors [3,40,42]. The −35 hexamer in the promoter and its just upstream *cis*-element are required in vivo for light-responsive K-81 *psbA2* transcription [22,23]. Therefore, there is a possibility of activity and/or structural changes to SigB/SigD caused by the redox signal with other *trans*-acting factors, which can confer the ability to enhance sigma binding affinity to the promoter or the core enzyme.

Another question is how the light-sensing signal transduction pathway, involving changes in redox potential, is linked to the light-responsive transcription with sigma factors. Recent experiments revealed that osmotic responsive *sigB*/*sigD* and cold-induced *sigD* transcript levels were reduced in ΔHik33 (*sll0698*, a sensor histidine kinase) cells [43]. This indicates that the sensor protein also regulates *sigB* and *sigD* expression. Further study of the signal transduction pathway, via the redox state and/or a sensor(s), transferring signals for transcription will shed light on the mechanism of light- and dark-responsive gene expression in cyanobacteria.

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